

Combined chemo- and immunotherapy of tumors induced in mice by *bcr-abl*-transformed cells

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Abstract. For our experiments we selected two oncogenic, *bcr-abl*-transformed mouse cell lines, viz. B210 and 12B1. Both cell types are capable of inducing leukemia-like disease in syngeneic BALB/c mice after intravenous inoculation. 12B1 cells can moreover form solid tumors after subcutaneous injection. Since immunotherapy would expectedly be most effective in animals in which the tumor mass had been reduced by other therapeutic means, we attempted to develop a combined therapeutic system for suppressing tumor growth. In the present study, mice inoculated with the aggressive 12B1 cells were treated with imatinib mesylate (IM), mouse interferon α (IFN α) and cyclophosphamide (Cy) in combination with genetically modified tumor cells engineered to produce various cytokines. These cell vaccines had been derived from B210 cells. Therapy with IM or IFN α alone or cell immunotherapy alone resulted in partial suppression of tumor growth. Of the different therapeutic regimens tested, a combination of repeated doses of IM, IFN α and cell vaccines with one relatively high dose of Cy (200 mg/kg) was the most effective, resulting in tumor-free survival of a large portion of mice. The spleens, livers and bone marrows of the successfully treated animals were tested for the presence of *bcr-abl*-positive cells by means of RT-PCR technique. Results were negative, this suggesting that the animals had been cleared of residual disease.

Introduction

Chronic myeloid leukemia (CML) is a lethal disease of blood stem cells. In the pathogenesis of this disease, the key role is played by the *bcr-abl* fusion gene, which originates from a translocation between chromosomes 9 and 22. The product of the fusion gene exists in three forms, viz. p210^{bcr-abl}, p190^{bcr-abl}

or p230^{bcr-abl}. Of these, p210^{bcr-abl} is the most common. The *bcr-abl* fusion protein has a markedly increased activity of tyrosine-kinase (TyKi), which is coded for by the SH1 domain of the *abl* gene. It is generally accepted that this activity is responsible for both the cell transformation and the maintenance of the transformation state (1,2).

Targeting the TyKi activity of *bcr-abl* appears to be a highly attractive therapeutic strategy (3). Imatinib mesylate (IM), one of the 2-phenylaminopyrimidine derivatives, is a direct inhibitor of the TyKi activity of the BCR-ABL fusion protein (4-6). IM competitively inhibits the interaction of these proteins with adenosine triphosphate (ATP) (7) which is necessary for TyKi activity. IM inhibits the growth of *bcr-abl*-positive cells both *in vivo* and *in vitro* (8-10) and is capable of inducing long-term remissions and prolonging the life of CML patients considerably (11-13). In the treatment of chronic phase CML, IM has provided much better hematological and cytogenetic responses than IFN α (14), which until recently was widely used for CML treatment. Already the early studies in mouse model systems also proved a high efficacy of IM, viz. retardation of the growth of tumors induced by *bcr-abl*-transformed cells (4,9,15). In the past few years several mechanisms of resistance to IM have been recognized (13,15-17). Furthermore, in spite of its high specificity and low toxicity, some dose-dependent side effects of IM have been reported (12,18). Quite recently impairment of proliferation and function of CD4⁺CD25⁺ cells (19) and a strong, though transient anti-leukemia immune reaction (20) have been observed in IM-treated patients. On the basis of the experience with this drug, combination of IM with other antileukemic agents has been proposed and examined. Burchert *et al* (21) have reported that the concurrent or sequential combinatory therapy with IFN α and IM, taking advantage of their different effector mechanisms, has been more effective in the treatment of CML than any current monotherapy. Also our results (Sobotkova *et al*, unpublished data) indicated that a combination of IM with IFN α was more effective in suppressing leukemia-like disease induced by the *bcr-abl*-transformed Ba-P210 (B210) cells developed by Daley and Baltimore (22) than its treatment with either of these substances alone (23). A combination of IM with IFN α and other substances *in vitro* has resulted in additive or synergistic antiproliferative effects, with *bcr-abl*-positive cell lines having been used (24-26). For example, Kano *et al* (25) have shown some synergistic *in vitro* cytotoxic effects of IM

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and recombinant IFN α and an additive effect of 4-hydroperoxy-cyclophosphamide [the active form of cyclophosphamide (Cy)]. Because of the immunomodulatory activity of Cy (27-30) one could expect additional beneficial *in vivo* effects of this drug if used either alone or in combination with the above drugs and/or with experimental vaccines.

In the study reported below, the therapy of disease induced in mice by *bcr-abl*-transformed cells was attempted with IM, IFN α and Cy, applied either alone or in various combinations and in combination with vaccines based on another syngeneic *bcr-abl*-transformed cell line that had been engineered to produce either interleukin-2 (IL-2) or interleukin-12 (IL-12) or the granulocyte-monocyte colony-stimulating factor (GM-CSF). To be able to easily reveal possible synergistic effects, we employed suboptimal treatment regimens with IM and IFN α .

Materials and methods

Cell lines and media. Two BALB/c mouse cell lines transformed by the *bcr-abl* gene (b3a2) and expressing p210^{bcr-abl} protein were used. 12B1 cells were obtained through the courtesy of E. Katsanis (University of Arizona, Tucson, AZ, USA). They had been derived by transformation of primary bone marrow cells with a retrovirus-derived vector carrying the *bcr-abl* fusion gene (31). Ba-P210 (B210) cells were kindly provided by G.Q. Daley (Whitehead Institute of Biochemical Research, Cambridge, MA, USA). They had been derived from interleukin 3 (IL-3)-dependent Ba/F3 cells (22). Their transduction by the *bcr-abl* gene carried by a retroviral vector had made them IL-3-independent. We described the basic *in vitro* and *in vivo* characteristics of these two cell lines in more detail elsewhere (10,32). In brief, both express approximately the same amounts of the p210^{bcr-abl} protein but differ in MHC class I expression (it is down-regulated in B210 cells) and in oncogenic potential. While B210 induce leukemia-like disease at doses exceeding 5×10^4 cells only after intravenous (i.v.) inoculation, 12B1 induce leukemia at doses $< 10^2$ cells after i.v. inoculation and, in addition, induce solid tumors after subcutaneous (s.c.) inoculation of doses equal to or $> 10^3$ cells. These tumors exhibit a high metastatic activity to spleen, liver and bone marrow. Both cell lines are highly susceptible to IM *in vitro*. After i.v. inoculation of 5×10^5 B210 cells, the animals develop leukemia in the course of the third week. After s.c. inoculation of 5.10^3 12B1 cells, animals form rapidly growing solid tumors that appear after 12-14-day incubation. Bcr-abl-negative HL-60 cells were used as a negative control in PCR (see below). All cell lines were passaged in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% FCS (PAA Labs., Linz, Austria), 2 mmol/l glutamine and antibiotics. In the case of 12B1 cells, the medium was furthermore enriched with 1 mmol/l sodium pyruvate and 50 μ mol/l 2-mercaptoethanol.

Cell vaccines. After repeated passages of B210 cells in the presence of increasing concentrations of 5-bromo-2'-deoxyuridine (BUDR), a thymidine-kinase-less (TK⁻) subline was isolated. These cells, designated B210TK/cl-2, grew well in the presence of 100 μ g BUDR, but did not grow in medium supplemented with hypoxanthine, aminopterin and thymidine

(HAT media Supplement, Invitrogen, Carlsbad, CA, USA) and were oncogenic for mice after i.v. inoculation. After transfection with bicistronic plasmids carrying genes for herpes simplex virus TK (HSV TK) and for various cytokines, genetically modified cell lines were isolated in HAT media. The construction of these plasmids was described previously (33). In addition to HSV TK, the gene-modified cells expressed IL-2 (B210/IL-2, IL-2 production 46.5 ng/10⁶/24 h), IL-12 (B210/IL-12, IL-12 production 130 ng/10⁶/24 h) or GM-CSF (B210/GM-CSF, GM-CSF production 40 ng/10⁶/24 h). *In vitro*, all three cell lines were highly sensitive to ganciclovir, this confirming HSV TK production, and were non-oncogenic after i.v. inoculation (Petrackova *et al.*, unpublished data).

Animals used and tumor induction. Seven- to 8-week old BALB/c female mice were used in all experiments. They had been obtained from Charles Rivers, Germany. The mice were inoculated s.c. with 5×10^3 12B1 cells in 0.2 ml PBS. In therapeutic experiments, 10^6 B210/IL-2, B210/IL-12 or B210/GM-CSF cells in 0.2 ml PBS were repeatedly injected intraperitoneally (i.p.) (see the Results section). The animals were inspected for tumor development at least twice a week and tumor size was measured with a caliper. Animals carrying tumors exceeding 20 mm in their longest diameter were humanely sacrificed. All animal studies were done in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic.

Reagents. Imatinib mesylate (IM, STI571, Glivec) was a generous gift from Novartis (Basel, Switzerland). It was dissolved in distilled water (1 mg/ml), sterilized by filtration through a Millipore filter, distributed into vials and kept at -20°C until use. It was inoculated i.p., one dose (50 mg/kg) per day, five times a week for one or two weeks, starting on day 3 or 10 after the s.c. inoculation of 12B1 cells. Cyclophosphamide (Cy) (Farnos, Finland) was given in a single i.p. dose, 200 mg/kg, on day 3 after inoculation of 12B1 cells. Recombinant mouse interferon α (IFN α) (Calbiochem, Merk Biosciences, Darmstadt, Germany) was administered starting on day 3 or day 10, one dose (1000 IU) per day, five times a week, for one or two weeks.

PCR used for detection of *bcr-abl*-positive cells in animal tissues.

Sampling. Livers and spleens were taken from a portion of the mice surviving the treatment without tumor development and from some tumor-bearing animals. The tissues were immediately frozen in liquid nitrogen and stored at -70°C until examination. Bone marrow was also sampled from some animals. It was obtained by irrigating tight bone with 1 ml of sterile PBS. Cells were counted with a haemocytometer. RNA extraction (see below) was done immediately after collection of cells. Similarly treated organs from diseased animals served as positive controls.

RNA extraction. Prior to RNA extraction, the samples (250 mg of thawed liver or spleen tissue, or $4-10 \times 10^6$ bone marrow cells) were homogenized with a TH 220 hand-held homogenizer with disposable tips (Cole Parmer, IL, USA) in

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Reverse transcription. DNase-treated RNA (2 μ g) was reverse transcribed using an oligo (dT)₁₈ primer, 50 U MMLV reverse transcriptase (Promega, Madison, WI, USA) and 20 U Rnasin (Promega) in a total volume of 20 μ l. After an initial denaturation of RNA at 70°C for 10 min, the master mix was placed on ice and the sample was incubated at 37°C for 60 min.

PCR. The quality of cDNA was assessed by PCR with primers specific for the house-keeping β -actin gene (forward 5'CCACTGGGACGACATGGAGAAGAT3'; reverse 5'CATGGCTGGGGTGTGAAGGTC3'), which amplify the 166-bp-long fragment. The expression of the *bcr-abl* gene was monitored by means of nested PCR with a set of external primers that amplify the 327-bp-long fragment (forward 5'TTCAGAAGCTTCTCCCTG3'; reverse 5'CTCCACTGGC CACAAAAT3') and a set of internal primers that amplify the 245-bp-long fragment from the b3a2 spliced gene (forward 5'GTGAAACTCCAGACTGTC3'; reverse 5'CAACGAAAA GGTGTTGGGGT3'). Fifty microlitres of the first reaction mixture contained 5 pM/ μ l of each primer, 1.5 mM MgCl₂, 2 mM dNTPs, 0.5 U of Taq polymerase (Fermentas, Vilnius, Lithuania) and 1X PCR buffer with (NH₄)₂SO₄ (Fermentas). For the second PCR, 1 μ l of 10-fold diluted PCR product of the first reaction was used. Initial denaturation at 94°C for 5 min was followed by 35 cycles, each consisting of 1 min at 94°C, 90 sec at 57°C, 90 sec at 72°C and a final extension at 72°C for 7 min. The reaction mixture and cycling conditions for the second PCR were the same as for the first one. As a positive control, RNA extracted from B210 cells, and as a negative control, RNA extracted from HL60 cells, was used.

Statistical analysis. Tumor development was analyzed in 2x2 contingency tables by the two-tailed Fisher's exact test. For analysis of the growth curves of the tumors, the two-way analysis of variance was used. Calculations were done using the Prism Software Version 3.0 (Graph-Pad Software, San Diego, CA). A difference between groups was considered significant at $p < 0.05$.

Results

Effects of Cy, IM and IFN α on the growth of 12B1-induced tumors. Animals inoculated s.c. in the right back with

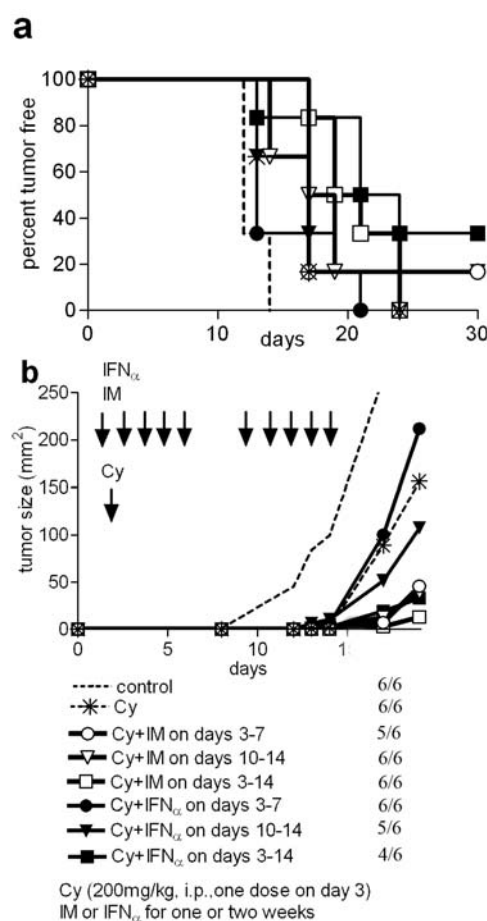


Figure 1. Effect of Cy, IM and IFN α on the growth of tumors induced by 12B1 cells. (a) tumor development, (b) tumor growth. The measurement of tumor size was terminated when it reached or just exceeded 20 mm in its longest diameter. Arrows indicate the days after 12B1 cell inoculation when the drugs were administered.

5x10³ 12B1 cells were treated with Cy (200 mg/kg) alone or in combination with either IM (50 mg/kg/day) or IFN α (1000 IU/day). Cy was administered in a single dose on day 3, while the other substances were given once a day for 5 or 10 days starting on day 3 or day 10, after the cell administration. The results of a representative experiment are shown in Fig. 1. It is evident from Fig. 1a that although nearly all mice developed tumors before the end of the observation period, Cy given alone or in combination with either IM or IFN α induced a considerable delay in their appearance. This delay was statistically significant ($p < 0.02$) in all the combinations tested except that in which IFN α was given on days 3-7. Fig. 1b shows that tumors grew at a much slower rate in mice treated with the drug combinations, more so in those treated with Cy in combination with IM ($p < 0.001$) than with IFN α (non-significant) for the early or late treatment and $p < 0.01$ for the early+late treatment. Furthermore, treatment with the Cy plus IM combination was significantly more efficient than treatment with Cy alone ($p < 0.01$). Similar results were obtained in repeated experiments.

Effect of cell vaccines producing IL-2, IL-12 or GM-CSF on the growth of 12B1-induced tumors. Animals inoculated s.c. with 5x10³ 12B1 cells on day 0 were repeatedly i.p. injected

Table I. Presence of *bcr-abl*-positive cells in treated and untreated mice inoculated with 12B1 cells.

12B1 cells	Therapy	Tumor present	Day of sampling ^a	Bone marrow	Spleen	Liver
i.v.	-	+	12	2/2 ^b	2/2	2/2
	-	+	18	NT ^c	2/2	2/2
s.c.	-	+	15	NT	3/3	3/3
s.c.	Cy+IFN α +B210/IL-2	-	124	0/4	0/4	0/4
s.c.	Cy+IM+B210/IL-2	-	123	0/2	0/2	0/2
s.c.	Cy+B210/IL-12	-	123	0/1	0/1	0/1
s.c.	Cy+IFN α +B210/GM-CSF	-	123	0/1	0/1	0/1

^aNumber of positive animals/number tested in RT-PCR. ^bAfter 12B1 cell inoculation. ^cNot tested.

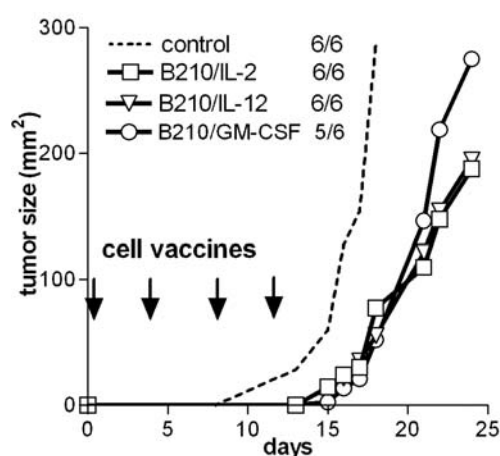


Figure 2. Effect of live B210 cell-based vaccines expressing different cytokines on the growth of tumors induced by 12B1 cells. Arrows indicate the days after 12B1 inoculation when the vaccines were administered.

with B210-cell vaccines producing IL-2, IL-12 or GM-CSF (10^6 cells per dose). The vaccines were given on days 0, 3, 7 and 10. It is evident from Fig. 2 that the therapy did not prevent tumor development, but it significantly postponed the appearance of tumors as compared with control animals ($p < 0.01$ in the case of all three vaccines). The tumors grew at a somewhat slower rate in animals injected with either the IL-2 or IL-12-producing cells than in those injected with GM-CSF-producing cells; however, 1 of the 6 animals in the latter group survived without tumor formation.

Effect of cell-vaccine therapy combined with Cy, IM and IFN α administration on the growth of tumors induced by 12B1 cells. Having obtained some basic information as to the effectiveness of the drugs tested and evidence that the cell-based vaccines can produce some, though a limited effect on 12B1 tumor formation, we decided to combine the two approaches. In the ensuing experiment the cell vaccines were administered in the same way as previously. A single dose of Cy was i.p. injected on day 3, either alone or in combination with the repeated administration of IM and/or IFN α . The latter substances were injected on days 3 to 7 and 10 to 14. The amount of each drug per dose was the same as in the preceding experiments. Results are shown in Fig. 3.

It can be seen that the administration of Cy alone resulted in a significant postponement of tumor appearance ($p < 0.05$), but did not prevent tumor formation in any of the animals. A similar effect was observed in animals treated with mixtures of Cy and either IM or IFN α or both. Animals which received Cy and any one of the three vaccines also responded similarly. However, when the combined Cy+cell-vaccine treatment was supplemented with either IM or IFN α in the case of the B210/IL-2 vaccine (Fig. 3a and b), or with IFN α in the case of the B210/IL-12 vaccine (Fig. 3c and d), tumors appeared later and grew slower ($p < 0.001$) than in the non-vaccine-treated animals. Most importantly, some animals did not develop tumors at all. This was most frequent in those treated with the B210/IL-2 vaccine. Two of the 6 mice which, in addition to the Cy+cell vaccine, also received IM, and 4 of the 6 animals which received IFN α in addition to the Cy+vaccine, remained tumor-free until the end of the observation period. The protective effects of the B210/IL-2 and B210/IL-12 vaccines administered in the above combinations were significantly higher than in the groups treated with Cy alone ($p < 0.01$, < 0.001 and < 0.02 , respectively). On the other hand, no beneficial effects of the B210/GM-CSF vaccine were apparent (Fig. 3e and f).

The vaccine treated mice surviving tumor-free for four months were tested for the presence of *bcr-abl* transcripts in cells obtained at autopsy from their livers, spleens and bone marrows. The results presented in Table I testify to the absence of leukemic cells in all of the surviving animals. On the other hand, both control animals with 12B1-induced tumors possessed *bcr-abl*-positive cells in the organs tested, this indicating a high propensity for metastizing of these tumors.

Discussion

The recently enhanced interest in the combination of chemo- and immunotherapy of CML is paralleled by an increased interest in suitable animal models, in which specific immune responses to *bcr-abl*-positive cells can be studied much more easily than in human patients. In the study reported herein, we used 12B1 mouse cells, which carry the *bcr-abl* gene and express the p210^{bcr-abl} protein and the drugs most widely used for the treatment of human CML, i.e. IM (now) and IFN α (until recently). The treatment regimen with these substances was intentionally suboptimal, because we expected that under these

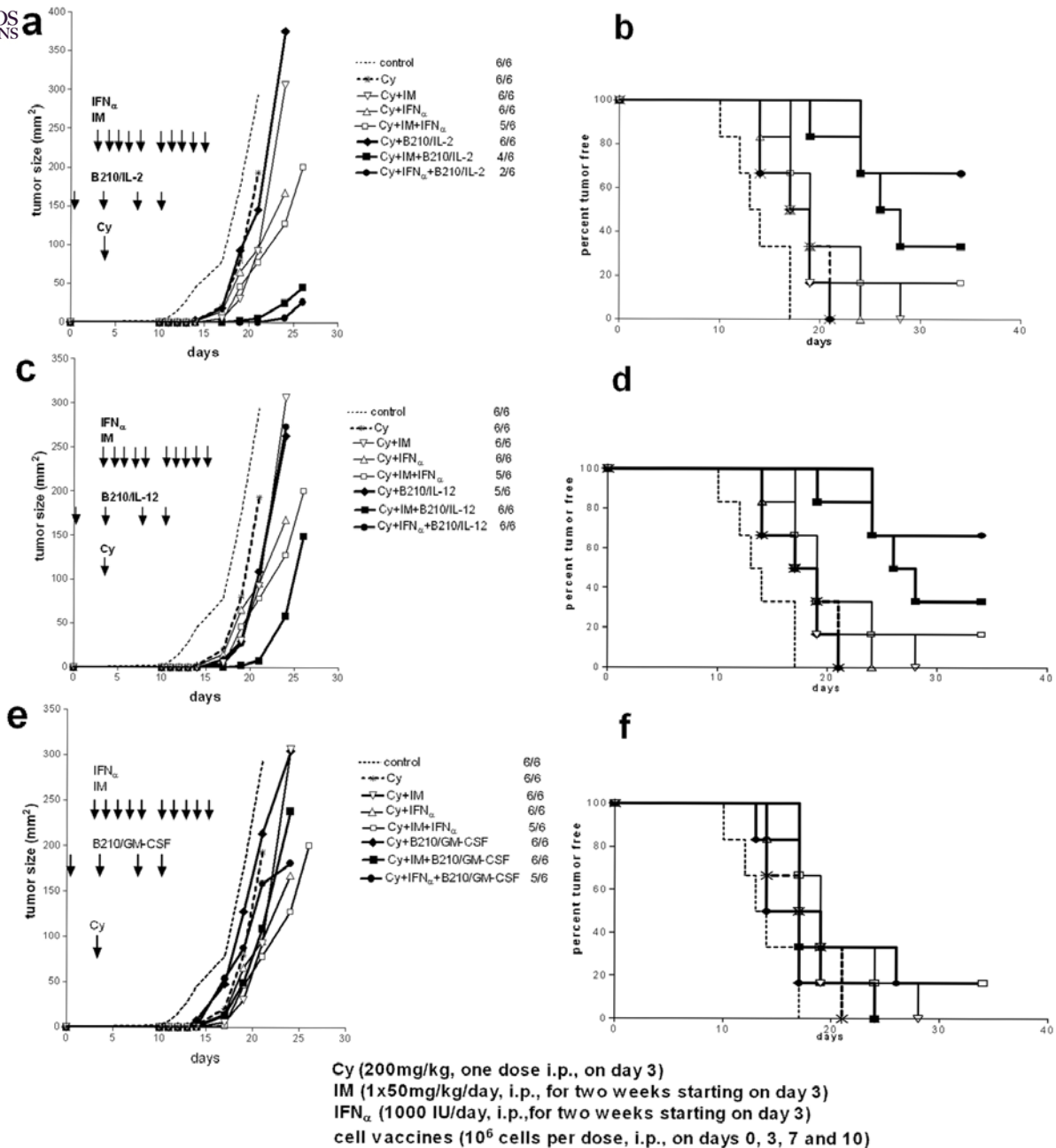
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Figure 3. Effect of therapy with Cy, IM and/or IFN α combined with live B210 cell-based vaccine administration on the growth of tumors induced by 12B1 cells. (a and b) Treatment with B210/IL-2 vaccine, (c and d) treatment with B210/IL-12 vaccine and (e and f) treatment with B210/GM-CSF vaccine. Arrows indicate time after 12B1 cell inoculation when the drugs and the vaccines were administered.

conditions any possibly synergistic effects would be more evident. These drugs were supplemented with Cy and with specific vaccines based on heterologous but syngeneic B210 cells, which also express the p210^{bcr-abl} protein and, in addition, several immunostimulatory factors known from many previous studies to enhance anti-tumor reactions.

In agreement with our previous results obtained with B210 cells (Sobotkova *et al*, unpublished data) and with results obtained in patients with CML (21,34-36), combinations of two or more of the above-mentioned drugs increased their effectiveness in the treatment of tumors induced by 12B1 cells. The mutual interactions of these substances are not quite clear at this writing. While the mechanism of action of IM is understood well, this is not so with the other two agents used.

It is believed that the beneficial effects of IFN α in the treatment of CML are mediated by both its immunomodulatory and antiproliferative effects (37-39). Similar effects can be expected in the case of Cy. Although this substance has been extensively used as a cytostatic drug in cancer treatment, recent evidence has made it clear that it suppresses regulatory T cells (28,30,40,41) and may have some other beneficial immunomodulatory effects (42-44). In those experiments lower doses of Cy (28) than in our present undertaking have been used. Thus it is likely that the antiproliferative effect of the substance played a major role in our system. This conjecture of ours is further supported by our earlier observation that a reduction of the Cy dose markedly decreased its effects (unpublished data).

The vaccines used in our present study were prepared with B210 cells transformed by the *bcr-abl* fusion gene and expressing the p210^{bcr-abl} protein. Gene-modified cells expressing IL-2, IL-12 or GM-CSF were employed. Since they were free of oncogenic potential (Petrackova *et al*, unpublished data), they were used for therapeutic vaccination as live cells. In the therapeutic regimen chosen, none of the vaccines was capable of completely suppressing tumor development. However, all three delayed tumor formation significantly. Hoping that the concurrent administration of the chemo- and immunotherapy would result in a synergistic effect, we combined the two therapeutic modes. The combination was indeed more effective than the drug therapy alone. The effects were most conspicuous with the B210/IL-2 vaccine and weakest with the B210/GM-CSF vaccine. Some of the animals treated with the vaccine-drug combinations survived without developing tumors. The livers, spleens and bone marrows of these animals were checked by RT-PCR for the presence of *bcr-abl*-transcripts. The results were negative, which indicated that these animals were free of *bcr-abl*-positive cells and could thus be considered free of residual disease.

Presumably, the different 'therapeutic' activities of the vaccines were a consequence of the biological activities inherent in the cytokines tested and they seem to be in line with the experience of other investigators as well as with our earlier observations in another system (45). Still, it is possible that also the amount of the cytokine produced played a role. Since live cells were used for the vaccination in our experiments, they continued to replicate *in vivo* for some time, with the replication rate, though limited, most likely varying between the cell lines tested and thus producing varying amounts of the immunizing antigens as well as of the cytokines. Experiments are under way in which cell vaccines derived from clones differing markedly in the production of GM-CSF are being tested.

At this writing, it is not understood which tumor-cell antigens are involved in establishing immunity to the highly aggressive 12B1 cells. Although we originally speculated that the new epitope carried by the fusion zone of the p210^{bcr-abl} protein might mainly be involved, and in fact quite convincing evidence obtained in another laboratory (46-49) strongly indicated that this was the case, the results we obtained with a variety of genetic vaccines based on the fusion zone have not yet confirmed this (unpublished data). On the other hand, we have obtained evidence that other regions of the p210^{bcr-abl} protein are able to induce protection against 12B1 cells (unpublished data). Furthermore, other antigens present in leukemic cells might also be involved. It has been reported that WT1 and proteinase 3 (Pr-3) are overexpressed in human leukemic cells and that it is possible to induce a beneficial immune reactivity in patients or experimental animals by peptide-based or other types of vaccines (50-53). Our attempts to demonstrate the presence of either WT1 or Pr-3 by using monoclonal antibodies against their mouse analogues have failed (unpublished data). However, it is conceivable that other proteins involved in the p210^{bcr-abl} activities or in the transformation procedure itself are overexpressed in either cell line and act as immunogens. Experiments aiming to identify at least some of the proteins involved are underway.

To summarize, combination of chemotherapy and immunotherapy with vaccines based on gene-modified cells expressing IL-2 or IL-12 prevented development of tumors in a significant portion of mice inoculated with the highly aggressive p210^{bcr-abl}-positive 12B1 cells.

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